

**Effects of gradual and sudden heat stress on seed quality of
Andean lupin, *Lupinus mutabilis*.**

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<p>Tiivistelmä — Referat — Abstract</p> <p>Andean lupin (<i>Lupinus mutabilis</i> Sweet) is a potential oilseed crop, with a very high protein content (40–45%) and 15–20% oil content. It is valued as an alternative protein source for both human and animal consumption, and like several other lupin species, has a potential role in phytoremediation. Previous experience in central and southern Europe has shown the crop to have low and unstable yields with high sensitivity to heat and drought during the grain-filling stage. The species may therefore be more suitable for cool-temperate climates, as it comes from similar altitudes and latitudes as potato. Therefore, an experiment was set out to quantify the responses of Andean lupin to heat stress, using both gradually and suddenly rising temperatures during grain filling.</p> <p>In preliminary tests 60 accessions from 4 germplasm banks were screened for time to flowering and daylength sensitivity. For this experiment, 3 accessions were chosen, 478435, 457972, 457977, with vegetative phase durations of 44, 53, 64 days from sowing to first flower, respectively. Forty two plants of each accession were sown and reduced to 30 on the basis of uniformity. Plants were grown in a glasshouse with 22 °C, 18 h days and 18 °C, 6 h nights until about 25 days after flowering. Ten plants of each accession were subjected to one of the following treatments: control (continuing in the same glasshouse conditions), sudden heat stress (transferred to a growth chamber and subjected to 38 °C from 11:00 to 15:00) or gradual heat stress (transferred to a growth chamber and subjected to temperature increases of 4 °C day temperature and 2 °C night temperature, with the final two days at 38 °C from 11:00 to 15:00). The plants were returned to the glasshouse and when mature, the seeds were harvested and pooled into 3 replicates per accession and treatment for quality analysis. Seed protein, oil, soluble sugar, ash and moisture content were determined. Data were calculated on the basis of percentage of overall seed mass and also on a milligrams per seed basis in order to reflect the seed physiology at grain-filling stage.</p> <p>Sudden heat stress had greater effects on seed composition than gradual heat stress. When compared with control, sudden heat stress resulted in more loss of every component than gradual heat stress, on a per-seed basis, in all 3 accessions and the responses of the accessions to the sudden stress were not statistically different. Under sudden heat stress, mean seed weight declined by 70%, protein content by 70%, oil content by 85%, ash content by 50%, and soluble sugar content by 75%. The accessions responded differently, however, to the gradual heat stress. Accession 478435 experienced significantly greater reduction in seed weight, protein and ash content than accessions 457972 and 457977. Oil content per seed and soluble sugar content per seed were also lower in 478435 than in the other two cultivars, but the difference was not significant. On the flour basis, sudden heat stress increased ash content and decreased oil content and soluble sugar content significantly in all accessions. Accession 478435 had highest value in ash content at significant level. Under gradual stress, protein and ash content were increased while oil mass and soluble sugar mass were decreased. 478435 had significantly higher protein mass and ash mass in flour with respectively 57% and 5.1%, 457977 had significantly higher soluble sugar content with 112 mg/g.</p> <p>The results showed that heat stress can have a significant effect on the quantity and quality of seed yield in Andean lupin. While all tested accessions were severely susceptible to sudden heat stress, gradual stress identified differences between accessions, with one being much more susceptible than the other two. The most susceptible accession was the earliest to flower. Gradual heat stress allows better resolution than sudden heat stress when screening germplasm for heat tolerance.</p>			
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Table of contents

1. Introduction.....	1
1.1 Effects of heat stress on seed composition.....	2
1.2 Adaptation to heat stress	4
2. Materials and Methods.....	8
2.1 Protein	9
2.2 Oil	9
2.3 Moisture	9
2.4 Ash	9
2.5 Soluble sugar.....	10
2.6 Reduction of oil, protein, soluble sugar, ash, moisture and weight of stressed plants from ones of control at per-seed level	10
2.7 Statistical analyses	10
2.8 Programs of control, gradual and sudden treatment	11
3. Results.....	12
3.1 Heat stress speeds up senescence.....	12
3.2 Total yield and seed weight	13
3.3 Protein content	15
3.4 Oil content.....	17
3.5 Ash mass	19
3.6 Soluble sugar mass.....	21
3.7 Moisture mass	23
4. Discussion	26
5. Conclusions.....	30
Acknowledgements.....	31
Reference	32
Appendix.....	35

1. Introduction

The Andean lupin, tarwi (*Lupinus mutabilis* Sweet), a legume originated from the Andean area of South America, is the only American species of the genus *Lupinus* that has been domesticated and cultivated. Its distribution ranges from Colombia to the north of Argentina. *L. mutabilis* is considered to be neutral to day length and has indeterminate growth. In some countries such as Peru, Ecuador and Bolivia, tarwi is valued as an important food source for both humans and animals. The total area of tarwi in these countries is estimated at 10,000 hectares (Jacobsen & Mujica, 2007).

Research has been carried out for 40 years with the Andean lupin in Peru and other Andean countries, and the germplasm collection in Peru consists of about 1,200 accessions. Andean lupin was reported to have vegetative period of 140–230 days, seed yield of 800–2,700 kg/ha, protein content of 40–45% and oil content of 15–23%, and was shown to be able to fix 160–220 kg/ha N through the symbiosis with rhizobia (Jacobsen & Mujica, 2007).

L. mutabilis has been introduced into Europe. In Germany, yield was 0.68–3.0 t/ha (Weissmann & Weissmann, 1992; Romer & Jahn-Deesbach, 1982), with 1.8–6.5 t/ha obtained in 1991 and 0.2–2.4 t/ha in 1992. In Spain, the yield was 0.2–0.5 t/ha (Lopez-Bellido, 1992). Yields of *L. mutabilis* in these countries were quite unstable and highly influenced by environmental factors (Jacobsen & Mujica, 2007). Its low yield, in part, is attributable to its low harvest index (HI) of only 0.09–0.33 (Hardy & Huyghe, 1997).

However, some valuable characteristics compensate and make *L. mutabilis* a valuable crop, its symbiotic relationship with rhizobia can provide N from air. The cross-pollination rate, on average, was 38%, ranging from 20%–80% depending on the populations, morphological characteristics were highly heritable and additively inherited. This offers a solution to obtain new cultivars through conventional breeding programme (Jacobsen & Mujica, 2007).

In Mexico, Norma et al. (2008) managed to use tarwi seed flour as an additive in making bread, after detoxification and decoloration. In detoxification and decoloration process, no harmful chemicals were involved. In the detoxification, seeds were soaked and boiled in water for 5 minutes and washed; and in decoloration, citric acid was employed which is not harmful to humans at low concentration (1%). As a result, unfavorable color was eliminated, and it further showed addition of 5% lupin flour or 2.5% lupin protein concentrate, or 0.5–1% lupin protein isolate could prolong the shelf life of loaf bread. Sensory tests by both trained and untrained judges proved pleasant acceptance.

Like several other lupin species, *L. mutabilis* has a potential as a part in phytoremediation. *L. albus* was adopted as a candidate in phytoremediation on some soil contaminated by heavy metals such as Cd, As, Zn, and Pb. Result showed heavy metals were reduced significantly (Peñalosa et al., 2007).

Since the yield of *L. mutabilis* is unstable in response to environmental factors, and its potential use in phytoremediation in contaminated soil or growth in other adverse conditions, it is necessary to investigate the response of *L. mutabilis* to stresses, especially the effects of stresses on the grain–filling stage, when drought and heat stresses are major limiting factors affecting the yield and quality of many crops. Furthermore, in contaminated soil, irrigation is usually not available.

The rate of climate change presses society to evaluate its consequences, respond and adapt. In recent years, average temperature has been rising along with fluctuations. If this trend is inevitable, to maintain or increase the current crop productivity, adaptation to climate change is necessary. It is predicted that crop productivity will fall in tropics and other areas where high temperature or inadequate rain limits crop productivity (Reddy & Hodges, 2000). Therefore, it is important to investigate the impacts of climate change on plant physiology, on yield and on quality.

1.1 Effects of heat stress on seed composition

Many studies about effects of high temperature on plant physiology and production have been conducted on some specific crop species in recent years. Spiertz et al. (2006) imposed heat shock in controlled growth chambers on different genotypes of wheat after flowering. Flour protein concentration (mg/g) from heat–stressed seeds increased in all of the genotypes and the mean kernel weight and yield were reduced. The reduction of yield and kernel weight under heat stress is due to inhibition of photosynthesis and respiration when the temperatures are above the temperature compensation point; finally, and CO₂ fixed by photosynthesis can not catch up with the carbon used as energy source for respiration. As a result, carbohydrate reserves such as starch decline. Starch accounts for most of the dry matter in storage organs such as cereal endosperms (Wardlaw, 1994).

Furthermore, Starch synthase was found to be inactivated due to its tertiary–structure change when the temperature is high enough to cause stress (Denyer et al., 1994). The grains of wheat plants grown at 21 °C lost more than 50% of the extractable activity of soluble starch synthase within 30 min of transfer to 37 °C (Rijven, 1986). Purified iso–forms of starch synthase from pea embryo were inactivated when the temperature was above 35 °C (Denyer & Smith, 1992). Because of the great loss

of carbohydrate caused by high temperature, the ratio between carbohydrate and oil, both and protein can be altered, resulting in quality change. Many studies have shown the effect of heat on the composition of grains and seeds.

Protein content is an important index that must be taken into account for both human and animal consumption. Many studies results have shown that the protein concentration of flour following high temperature stress of developing grains of wheat is greater than that of nonstressed (Stone, 1997; Spiertz et al., 2006). However, protein content per seed showed an opposite pattern, with lower values from stressed than non-stressed grains (Stone, 1996). Presumably, protein synthesis was also down-regulated by heat stress, but it was proportionally less affected than starch synthesis.

Oil, like starch, is stored energy used by seeds during germination. The effect of heat stress on seed oil production has been evaluated in a few studies. Oil concentration in canola (*Brassica rapus* L.) seeds showed a much lower value from the heat stressed plants (Aksouh et al., 2001). Fatty acid synthesis is based on two-carbon addition which uses acetyl-CoA as precursor, and acetyl-CoA is mainly produced from glycolysis, so photosynthesis inhibition by heat stress will lower down oil synthesis, for instance, Lipid synthesis in heat-shocked leaf materials of common bean (*phaseolus vulgaris* L.) was down-regulated. (Taiz & Zeiger, 2006).

The effects of heat stress on soluble sugar concentration of developing seeds are not well-known at present. Drought stress increased soluble sugar concentration in leaf materials of 49 pea cultivars, the authors suggested that this rapid accumulation preserved the assimilates for later seed filling in addition to protecting the leaves from water deficit (Sánchez et al., 1998). In heat-stressed plants, Photosynthesis is inhibited, so carbohydrate content should be decreased, but starch synthase and similar energy-storage enzymes are impaired, so small molecules may not be polymerized.

Heat increases rate of evaporation of water, so separating the effects of high temperature from those of water deficit requires careful experimentation. In study on the response of 2 wheat cultivars to sudden heat stress at grain-filling stage, the water content per kernel was lower in stressed grains than in control (Stone & Nicolas, 1995b). In the following study, water content per kernel was lower in both sudden and gradual heat stress treatments than in the control throughout the grain-filling stage of two wheat cultivars, and the water content at per-kernel scale was shown to be lower compared with control throughout the grain-filling stage (Stone & Nicolas, 1995a).

1.2 Adaptation to heat stress

As the climate is changing globally, mostly towards higher temperature and more erratic rainfall, high temperature affects the yield and quality, it is necessary to adapt crops to this change. In Australia where the weather is often hot and arid, many valuable studies on drought stress and heat stress has been done.

The first solution to some abiotic stresses such as drought and heat stresses is good infrastructure and facilities in the field. But this solution needs huge financial investment by the farmers and support from governments.

The second solution is to diversify agricultural production by introducing and domesticating some new species or varieties, as humans have done since agriculture began. During domestication, screening germplasm for resistance or tolerance to different stresses is very important. We should be optimistic that results of some studies showed a wide variation in resistance to heat stresses (Wardlaw, 1994; Stone & Nicolas, 1995a; Stone & Nicolas, 1995c).

In order to determine which line is more resistant or tolerant to a particular stress, standard and efficient screening technique must be established. In a review on screening techniques and sources of resistance to abiotic stresses in cool-season food legumes, the authors reviewed various abiotic stresses such as drought, heat, frost, chilling, waterlogging, salinity and mineral toxicities; and also some screening techniques such as leaf-water relations, osmotic adjustment, gas exchange, root characteristics etc. Some resistant or tolerant varieties were obtained based on above screening techniques (Stoddard et al., 2006). McDonald and Paulsen (1997) proposed a linkage between the sensitivities to drought and heat stresses and suggested that selection for one tolerance may improve the other. Selection of drought and heat-tolerant cultivars based on heat treatment alone may have the advantage that heat treatment can be regulated more easily and precisely than drought treatment, since temperatures can be more accurately set and readily adjusted than soil moisture content, thus reducing the noise in data analysis. To establish standard methods for heat tolerance, knowledge about the physiology under stress is necessary.

Taiz and Zeiger (2006) reviewed how plant physiology is influenced by heat stress and adapt to heat stress. Normally, shoots of most C_3 and C_4 plants with access to abundant water are maintained below 45 °C by evaporative cooling, but either because of low water availability or high atmospheric relative humidity, tissue temperature can increase, and then the plants are under stress. Besides the inhibition of photosynthesis, many other following physiological processes are affected and the plant

also can adapt itself to high temperature. Membrane stability was shown to be influenced by heat stress. Excessive fluidity of membrane lipids at high temperatures is correlated with loss of physiological function, and so is excessive rigidity at low temperatures. The degree of unsaturation of membrane lipids is one method by which plants can adapt to changing temperature and maintain membrane fluidity at an appropriate level, with more unsaturation required at lower temperatures and more saturation at higher temperature. There is a decrease in the strength of hydrogen bonds and electrostatic interactions between polar groups of proteins within the aqueous phase of the membrane at high temperature, thus modifying membrane composition and structure. Leakage of ions from the cell is widely used as a marker for inadequate cell membrane stability.

Plant, to protect leaves against excessive heating, includes some morphological changes and adjustment. For instance, both drought and heat tolerance depend on the same adaptations: reflective leaf hairs and leaf waxes; leaf rolling and vertical leaf orientation; and growth of small, highly dissected leaves to minimize the boundary layer thickness and thus maximize convective and conductive heat loss (Taiz & Zeiger 2006).

Transcription-factor-mediated HSP (Heat Shock Protein) accumulation is a protective mechanism of higher plants against heat stress. Heat stress causes many cell proteins that function as enzymes or structural components to become unfolded or misfolded, thereby leading to loss of proper enzyme structure and activity. Such misfolded proteins often aggregate and precipitate, creating serious problems within the cell. HSPs act as molecular chaperones and serve to attain a proper folding of misfolded, aggregated proteins and to prevent misfolding of proteins. This facilitates proper cell functioning at suddenly elevated, stressful temperatures (Taiz & Zeiger 2006).

Several signaling pathways mediate tolerance to heat stress. ABA (abscisic acid) and salicylic acid (SA) treatment can both lead to better survival under heat stress, while ABA- and SA-signaling mutants of *Arabidopsis* are both more sensitive to heat stress and unable to acquire thermotolerance. Ethylene biosynthesis is increased during heat stress, and ethylene pretreatment was shown to lower the thermotolerance of plants. Heat stress also causes oxidative damage to plant tissues. H_2O_2 can act as a signaling molecule increasing plant thermotolerance and leading to the production of HSPs. Calcium signaling appears to play a role in thermotolerance, but it is not well understood (Taiz & Zeiger, 2006).

Based on the above-mentioned, there have been some screening techniques developed in selecting heat-tolerant cultivars. In cowpea, heat tolerance was positively correlated with the membrane thermostability of leaf disks (Ismail & Hall, 1999), and with the harvest index and the percentage of

pod set (Ismail & Hall, 1998). Stone and Nicolas (1995c) selected several thermotolerant wheat cultivars from a set of 75 cultivars by evaluating the extent of variation in yield, a series of quality components such as grain nitrogen percentage, gliadin and albumin as a percentage of total SDS-soluble proteins, and noodle swelling power after imposing a heat treatment of 40 °C for 3 days. A study on the effects of high night temperature on rice also showed that the membrane thermostability was closely correlated with yield (Mohammed & Tarpley, 2009). Rahman et al. (2004) showed that the cotton seed yield was positively correlated with membrane thermostability when plants were heat-stressed.

Most studies on selecting heat-tolerance are based on analysis of membrane stability. Photosynthetic activity under stress was suggested in one study (Galiba et al., 1997), but this method is considered not feasible for broad screening due to its low maneuverability and high time consuming. It must be noticed that the above studies on the effects of high temperature are based on an arbitrary temperature, and usually on a sudden change of temperature, so it may be asked whether these experimental conditions are sufficiently representative of the real world, especially in temperate regions or high regions of high latitude. In the cotton study (Rahman et al., 2004), the susceptible cultivars generally produced more yield than heat-tolerant cultivars at moderate temperatures. Therefore, in crop production of temperate region or high latitudinal region, some cultivars with potential higher yield but less tolerance to this arbitrary high temperature could be eliminated in selection, so gradually-rising temperature design should be helpful in solving this problem.

Furthermore, Tripathy et al. (2000) found several QTLs that were associated with membrane stability in rice (*Oryza sativa* L.) under drought stress and suggested marker-assisted method can be useful in screening heat-tolerant lines. However, it is hard to generalize these markers to different plant species, so, it is necessary to correlate the phenotypic tolerance with tolerance-related alleles. The final purpose of screening heat-tolerant lines is to fulfill the requirements of the end consumers, so most of the studies finally define the tolerant cultivars by the variability in the qualitative components and yield under heat stress. Stone and Nicolas (1995c) screened some heat-tolerant cultivars of wheat based on some phenotypes such as the yield, composition of protein, and noodle quality made of flour from the experiment. Therefore, investigation between the quality, yield and other phenotypic and stress is the basis for further marker-assisted selection and acceptance from the end users.

As mentioned earlier, *L. mutabilis* has some attractive traits, and its production was shown unstable in southern and central Europe due to high temperature and drought, so it may be a suitable

candidate as an oilseed crop in high latitudes such as the the Nordic region. In the southern part of Finland, the suitable growing season is normally from May to August which is not long enough for some late varieties, so earliness of maturity is another critical factor, and in case of some adverse environmental factors, *L. mutabilis* could be used for phytoremediation where the irrigation system is often not available. Therefore, it is necessary to screen drought and heat tolerant cultivars with appropriate time for maturity and define a standard methods for selecting drought and heat tolerant cultivars.

In the Nordic region, it is not likely the temperature rises very sharply within a short period, so tolerance to such artificial conditions is not a desirable or necessary attribute. Gradually rising temperature in contrast could be a more useful method for screening heat tolerance. The objectives and hypothesis of this research are the following:

- ◆ How does the seed composition of *L. mutabilis* change under heat stress?
- ◆ Do accessions of different vegetative period vary in responding to heat stress?
- ◆ Does gradually rising temperature can increase the resolution in screening heat tolerance?

2. Materials and Methods

The whole experiment was conducted in Viikki campus, University of Helsinki, Helsinki, Finland (60°13' N, 25°02'E). Three accessions (three accessions were supplied by USDA, ARS, WRPIS Washington State University, Regional Plant Introduction Station, Table 1) were selected on the basis of their vegetative phase duration from preliminary screening of 60 accessions. PI457977, PI457972, PI478435, with 14 replicates of each accession for each of 3 treatments, were respectively sown on 20 November, 27 November, and 5 December in 2008 in order that flowering time would be synchronized. The potting mixture was composed of vermiculite and sand, with ratio 9 to 1. Plants were inoculated with an isolate of rhizobium from ElomestariTM (Elomestari company, Finland) and kept in a greenhouse with a 22 °C, 18-hour daytime, and 18 °C on 6-hour night. The 14 replicates of each accession were finally reduced to 10 replicates to ensure that plants under stress were at approximately same grain-filling stage. At 25 days after the onset of flowering (DAF), pods on the main stem of each plant were marked with a string. 10 plants of each accession were kept in the greenhouse as control (Figure 2.1A), 10 were assigned to the 'sudden' heat treatment (Figure 2.1B), 10 to the 'gradual' heat treatment (Figure 2.1C) by subjecting plants to growth chambers (). Plants of 3 accessions in chambers were randomized to avoid the effects being due to edge effect and well watered during the heat treatment. After the heat treatments, plants were returned to the greenhouse and allowed to grow to maturity. Seeds from the marked stem were harvested and weighed, and finally the 10 plants were pooled into 3 replicates (Seeds of 3 sets of 3 plants with the 10th plant were divided into 3 replicates), and the total weight of the replicate was determined. To determine average seed weight, a set of 50 seeds was counted and weighed.

Subsequently, seeds including their seed coat were milled to fine flour using an IKA mill (IKA LABORTECHNIK, Germany) for quality analysis as described below.

Table2.1: Cultivars, Origins and time from sowing to flowering

accession	Origin	Time (days) from sowing to flower (SD)
PI478435	Bolivia	44 (2.9)
PI457972	Peru	53 (3.1)
PI457977	Peru	64 (2.5)

2.1 Protein

200 mg of flour of each replicate from 3 treatments was subjected to the Dumas combustion (Marcó et al., 2002) method for nitrogen determination and N concentration was converted to protein concentration by multiplying by 6.25

$$\text{Protein content per seed} = \text{average seed weight} \times \text{Flour protein concentration} / 100$$

2.2 Oil

Oil was extracted by using petroleum ether (SIGMA, Germany) following standard protocols. About 2 g of flour, 3 steel balls, and 20 ml petroleum ether were put into a metal extraction tube, and that was then placed on a shaker. After 60 minutes at 175 rpm on the shaker, the mixture of petroleum ether and flour was transferred into tubes and centrifuged at 2000 rpm for 4 minutes. The supernatant was collected in pre-weighed vial and heated at 100 °C until the odour of solvent disappeared completely. Flour at the bottom of the centrifuge tube was transferred back to the extraction tube again for another round of extraction. The whole process was repeated twice. Oil mass was calculated according to the following formula.

$$\text{Oil mass} = \text{weight of vial with extracted oil} - \text{weight of empty vial}$$

$$\text{Flour oil concentration} = \text{oil mass} \div \text{weight of flour} \times 100$$

$$\text{Oil content per seed} = \text{average seed weight} \times \text{Flour oil concentration} / 100$$

2.3 Moisture

About 300 mg of flour was weighed into pre-weighed crucible; then flour with crucible was put into oven overnight at 105 °C, cooled down in dessicator and weighed. Moisture percentage was calculated according to the following formula.

$$\text{Moisture mass} = \text{weight of crucible with flour} - \text{weight of crucible and flour after heating}$$

$$\text{Flour moisture concentration} = \text{moisture mass} \div \text{weight of flour} \times 100$$

$$\text{Moisture content per seed} = \text{average seed weight} \times \text{Flour moisture concentration} / 100$$

2.4 Ash

About 200 mg of flour was weighed into pre-weighed crucible, then put into a muffle furnace at 600 °C furnace for 16–18 hours, cooled down in dessicator and weighed.

$$\text{Ash mass} = \text{weight of crucible and ash (after incineration)} - \text{weight of crucible and flour}$$

Flour ash concentration = ash mass ÷ weight of flour × 100

Ash content per seed = average seed weight × Flour ash concentration / 100

2.5 Soluble sugar

Soluble sugar mass was measured by the anthrone method (Sánchez et al., 1998). About 40 µg of defatted flour was weighed into microfuge tubes; then 2 ml of 70% ethanol was added. The tubes were vortexed, then were centrifuged at 10 °C, 2000g for 15 minutes. The supernatant was transferred into 10–ml test tubes. The whole process was repeated twice except for the third time, 2 ml deionized water was added into eppendorf tubes replacing 70% ethanol. 4 ml Chloroform was then added into the pooled supernatant; and each tube was swirled for a while to ensure chloroform and supernatant were well mixed. After 1 hour, the aqueous phase was collected for soluble sugar mass analysis.

50 µl of extract was added into 350 µl deionized water in microfuge tubes; and then 1.25 ml anthrone reagent was added and well mixed. Tubes were put into a water bath at 100 °C for 10 minutes and cooled to room temperature, and the solution was transferred into cuvettes; and analyzed by spectrophotometer at λ=630nm. Results were calculated based on the standard line constructed using pure glucose of different concentrations.

Seed sugar content = average seed weight × Flour sugar concentration / 100

2.6 Reduction of oil, protein, soluble sugar, ash, moisture and weight of stressed plants from ones of control at per–seed level

To determine how oil, protein, soluble sugar, ash, moisture and weight deviates from that of control at per–seed level, the percentage of reduced portion was calculated according to the following formula given that the grains from control are considered healthy.

Reduced portion = value of 3 replicates – mean of control

2.7 Statistical analyses

All the data were subjected to ANOVA using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) and analyzed by. Tukey test was hired for comparing means. Pearson correlation coefficients were also calculated.

2.8 Programs of control, gradual and sudden treatment

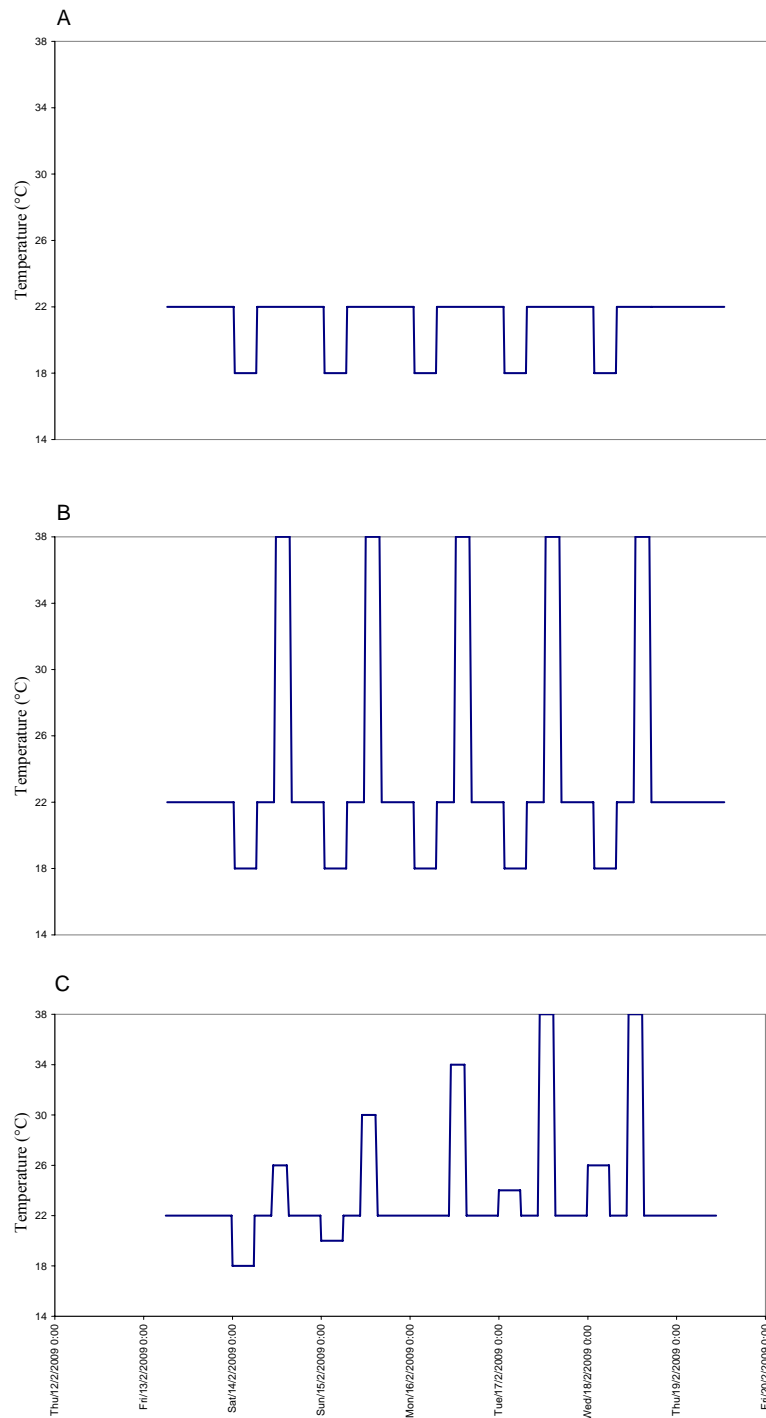


Figure 2.1. Temperature profiles showing (A) control treatment, (B) sudden treatment, and (C) gradual treatment

3. Results

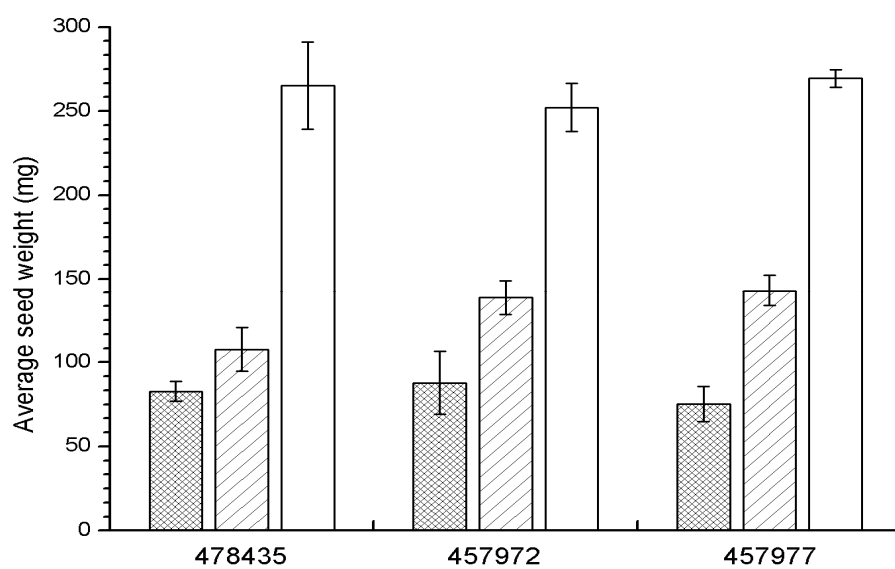
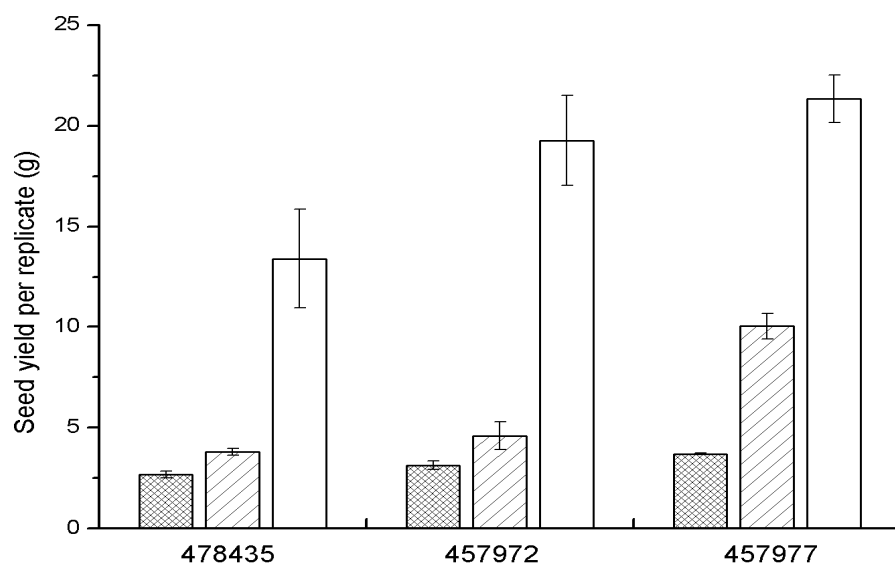
3.1 Heat stress speeds up senescence



Figure 3.1. Example plants, taken two days after completion of the heat stress treatment.

Heat stress caused the lower leaves to wither or fall, while leaves of control remained turgid and healthy.

3.2 Total yield and seed weight



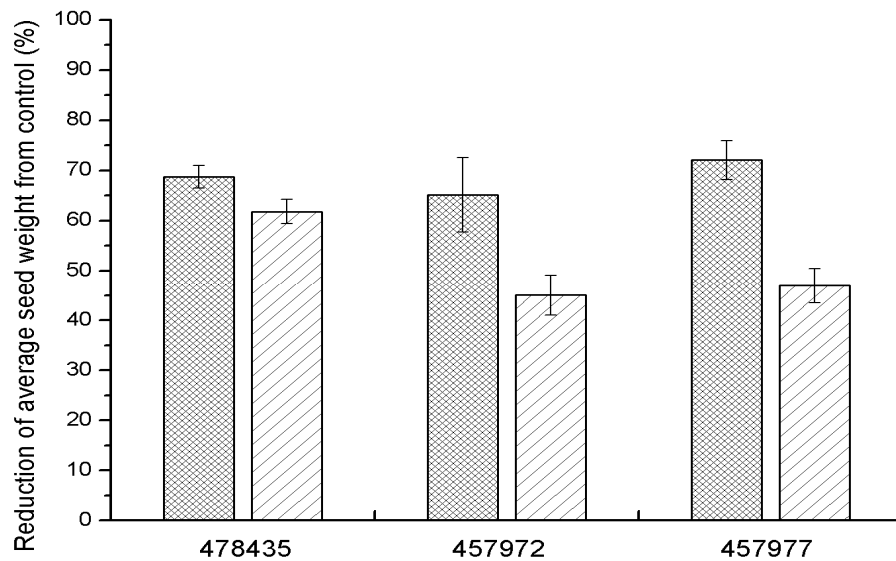
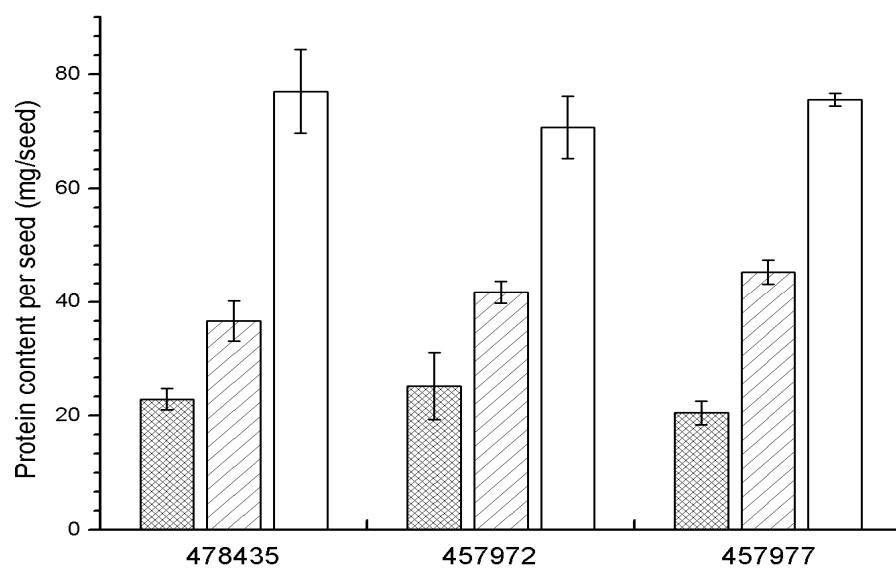
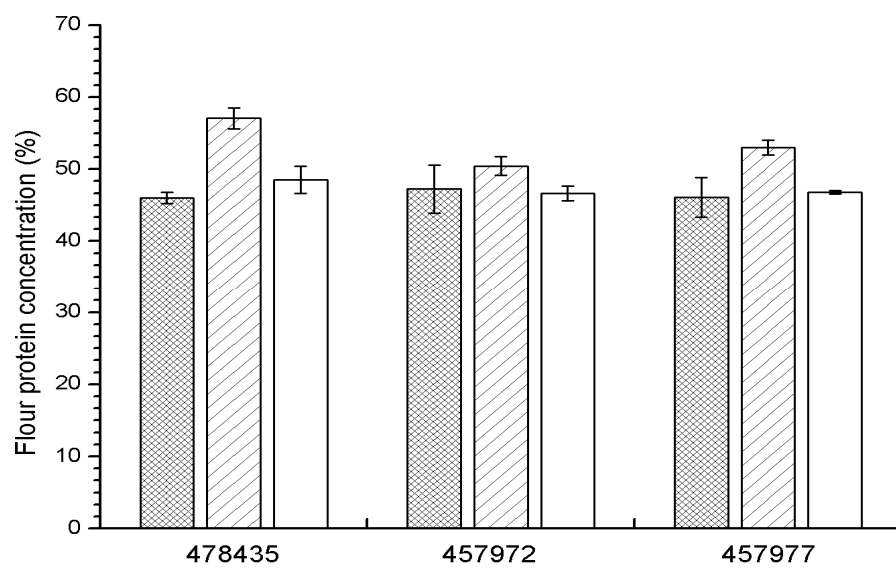


Figure 3.2. The effect of heat treatments on (A) seed yield per replicate, (B) average seed weight, and (C) the reduction of seed weight compared with control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

The yields of accessions 457972 and 457977 were about 20 g, significantly higher than the 13 g of accession 478435 ($P < 0.01$; Figure 3.2A). Heat stress caused great loss in both yield and average seed weight in all 3 accessions ($P < 0.01$). Yield and average seed weight were more reduced under sudden stress than under gradual stress. Under sudden heat stress, both yield and average seed weight were reduced to a level that was not significantly different between accessions, with yield and seed weight around 4 g and 80 mg respectively (Figure 3.2B).

Under gradual stress, yields of 457972 and 478435 were similar and lower than 457977; seed weight of 457972 was similar to 457977 and both were higher than 478435 ($P < 0.05$). Average seed weight of all 3 accessions under sudden stress were reduced by around 70%; however, under gradual stress, seed weight of 457972 and 457977 were reduced by around 45% while 478435 was reduced by 60% (Figure 3.2C). Notably, under gradual stress, although average seed weight of 457972 and 457977 was similar, 457977 was much higher yielding than 457972.

3.3 Protein content



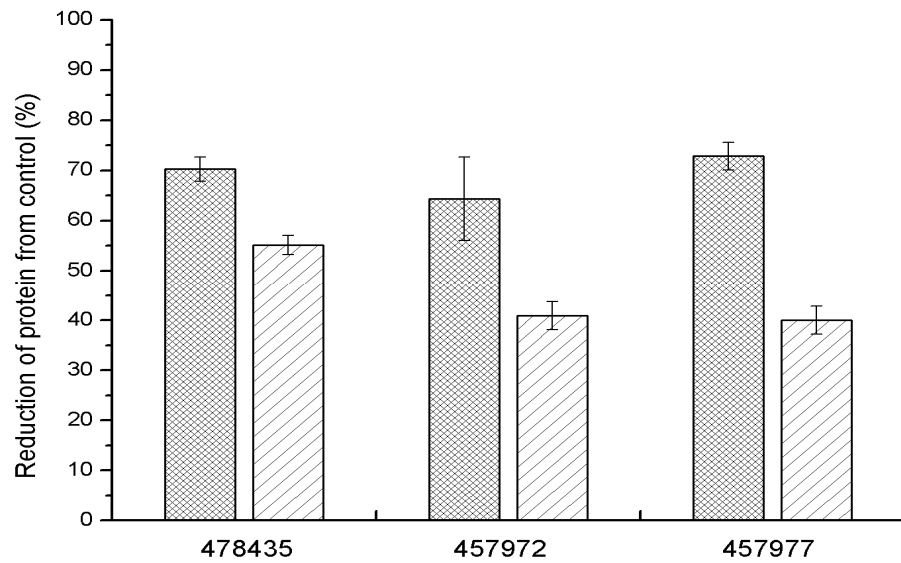
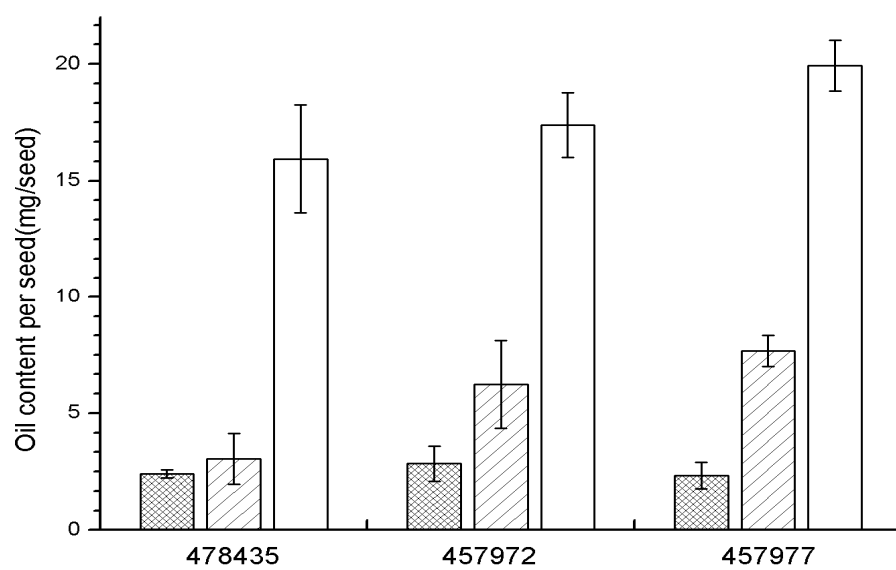
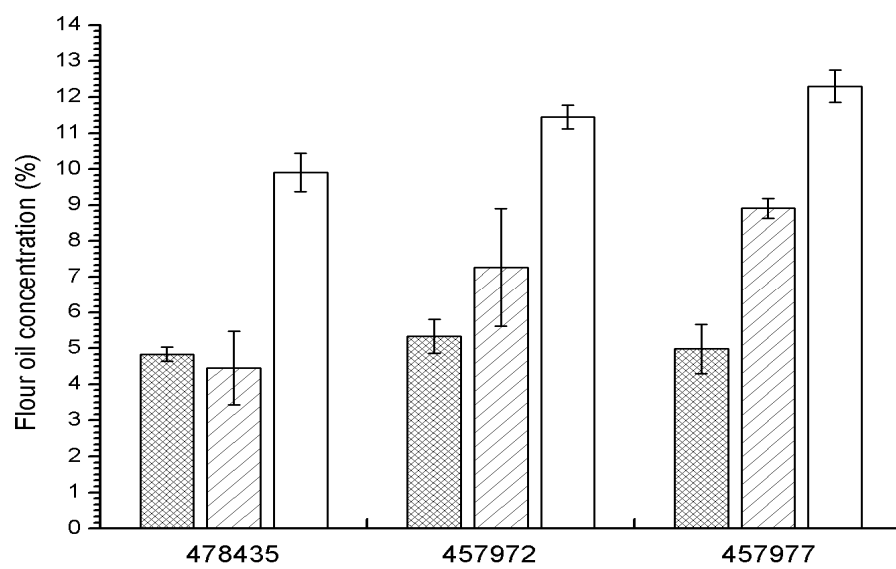


Figure 3.3. The effect of heat treatments on (A) flour protein concentration, (B) protein content per seed, and (C) the reduction of protein content from control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

Flour protein content was increased in all 3 accessions under gradual stress, and higher than both sudden stress and control ($P < 0.01$; Figure 3.3A). Seed protein content was highest in the control treatment, and seeds from gradual stress had higher protein mass than from sudden stress ($P < 0.01$; Figure 3.3B). The reduction of protein content per seed under sudden stress was about 70% and not significantly different between accessions, but under gradual stress, protein content in 457972 and 457977 was reduced by around 40%, while that of 478435 was reduced about 55%.

3.4 Oil content



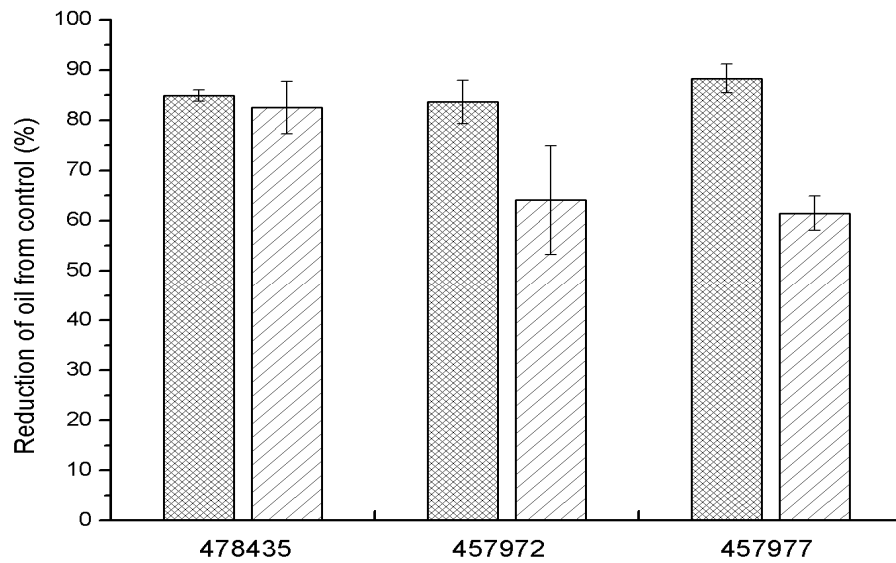
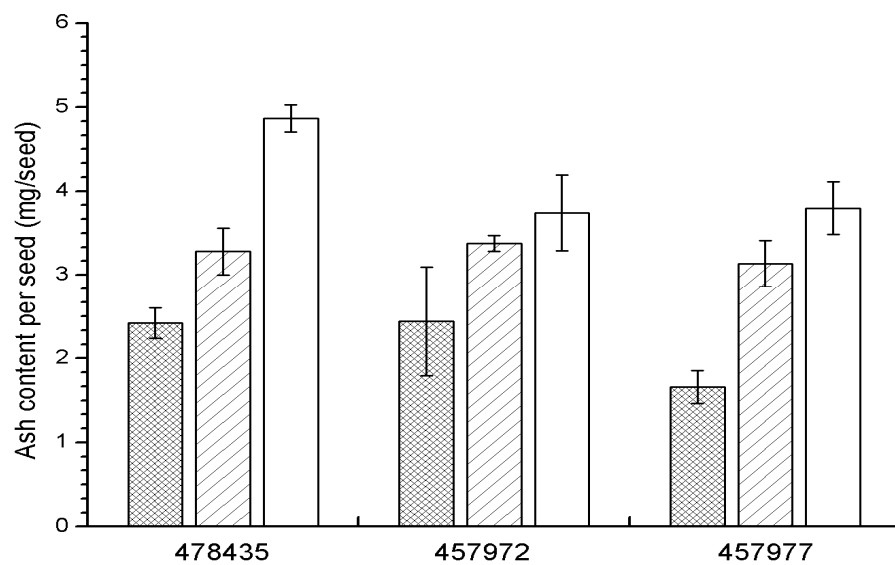
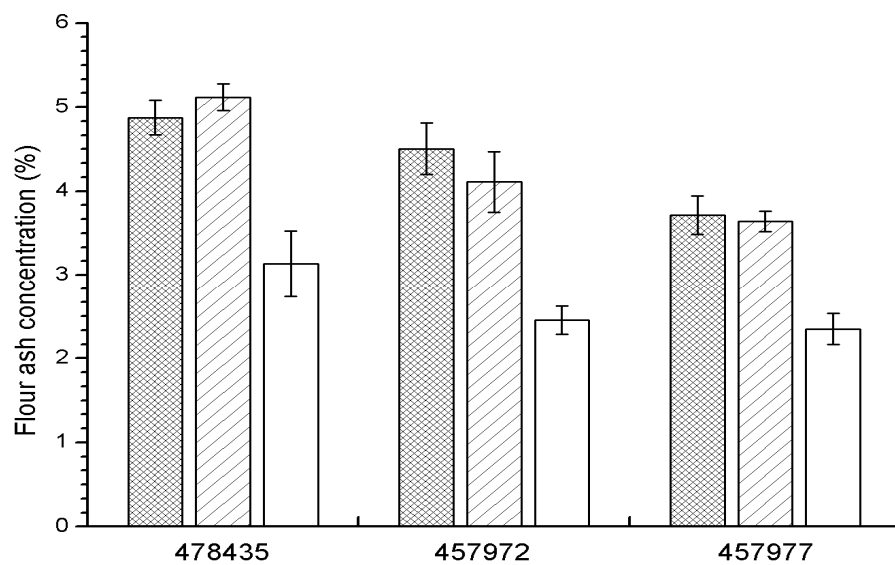


Figure 3.4. The effect of heat treatments on (A) flour oil concentration, (B) oil content per seed, and (C) the reduction of oil content from control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

Oil values showed similar patterns at both flour and seed levels; oil was reduced significantly in 3 accessions under heat stress ($P < 0.01$; Figure 3.4A and 3.4B). Sudden heat stress reduced oil more than gradual stress in accessions 457972 and 457977, whereas in 478435 sudden and gradual stress reduced oil by similar amounts. Sudden heat stress reduced oil per seed by about 85% in all 3 accessions (Figure 3.4C), whereas the effect of gradual heat stress was less in 457972 and 457977, although statistically not significant.

3.5 Ash mass



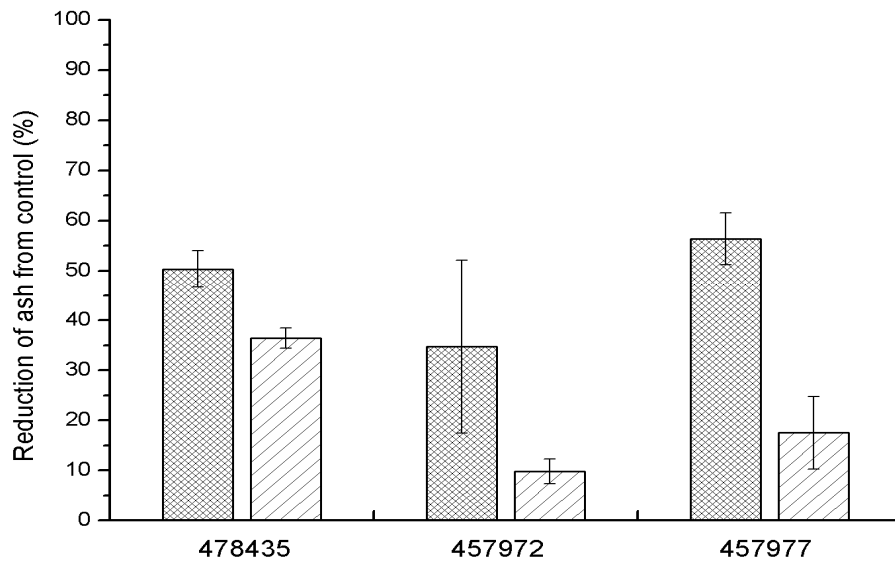
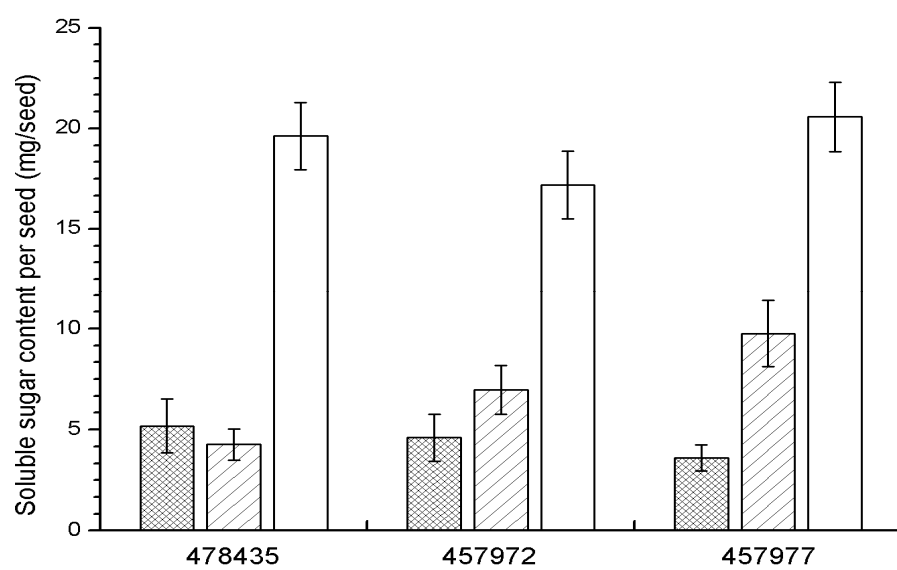
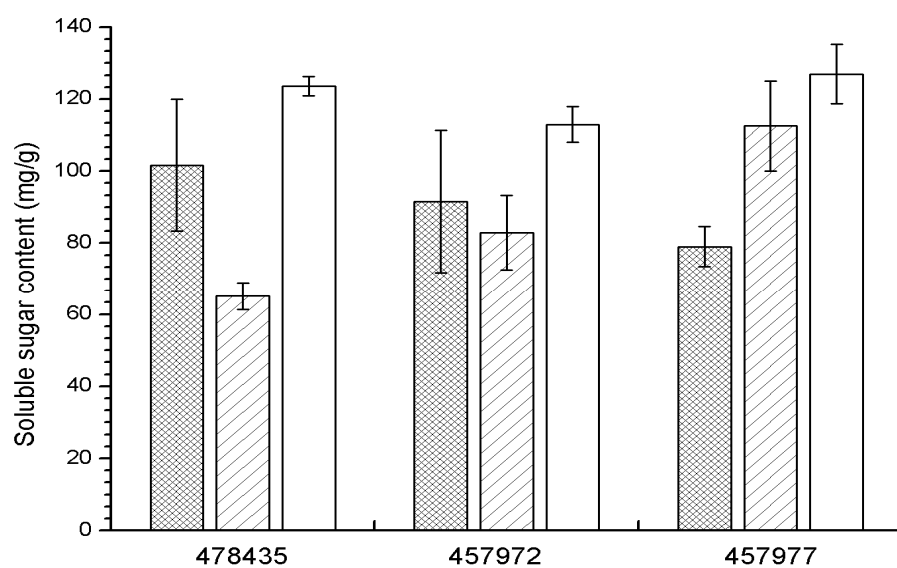


Figure 3.5. The effect of heat treatments on (A) flour ash concentration, (B) ash content per seed, and (C) the reduction of ash content from control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

Flour ash concentration increased in all 3 accessions under heat stresses to almost double that of control ($P < 0.01$; Figure 3.5A). Conversely, at the per seed level, ash content of control seeds was significantly higher ash than in heat-stressed ones; seeds from gradually stressed plants had higher ash than suddenly stressed ones ($P < 0.01$; Figure 3.5B). In control, 478435 had the highest value for ash mass at per seed level, but when under gradual stress, the ash content of seeds from 478435 was reduced most by around 35% whereas ash mass of 457972 and 457977 were reduced by about 15–20% ($P < 0.05$; Figure 3.5C). In sudden stress, ash mass was not significantly different across 3 accessions.

3.6 Soluble sugar mass



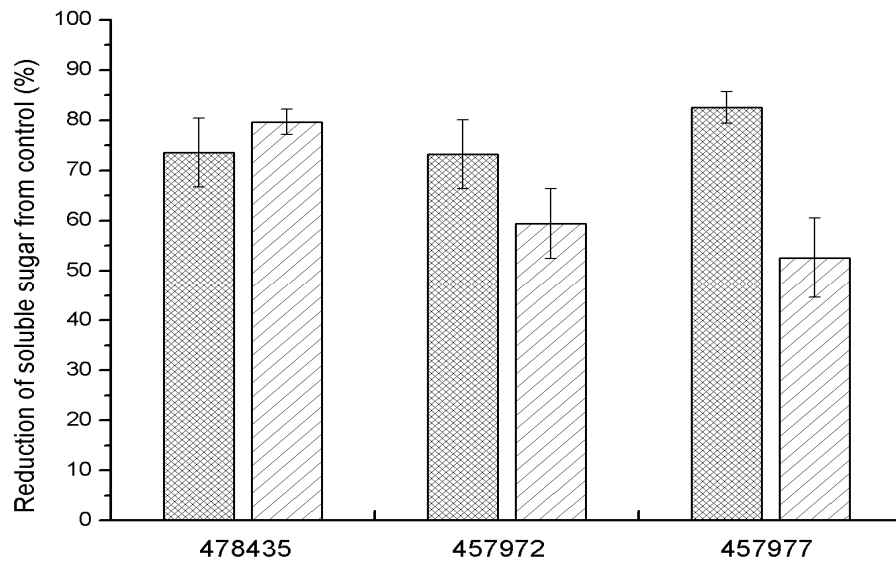
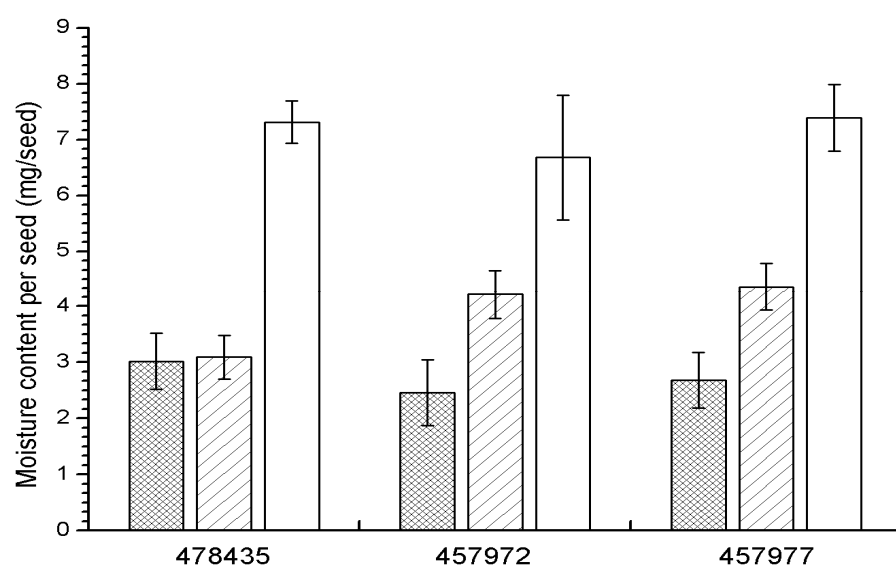
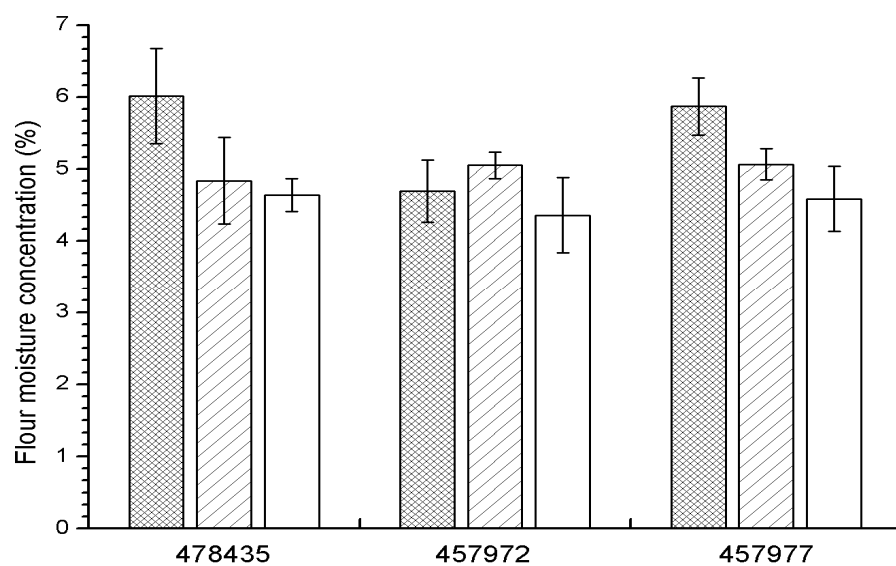


Figure 3.6. The effect of heat treatments on (A) soluble sugar content, (B) soluble sugar content per seed, and (C) the reduction of soluble sugar content from control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

Heat stress caused difference in soluble sugar mass in both flour (Figure 3.6A) and at per seed level ($P < 0.01$; Figure 3.6B). Both measures of sugar production were reduced under heat stress, especially, at per seed level, sudden heat stress decreased soluble sugar mass by around 75–80% in all 3 accessions compared with control (Figure 3.6C). In gradual heat stress, soluble sugar mass of 457972 and 457977 was reduced by 55% less than 478435 by 80% but not different significantly.

3.7 Moisture mass



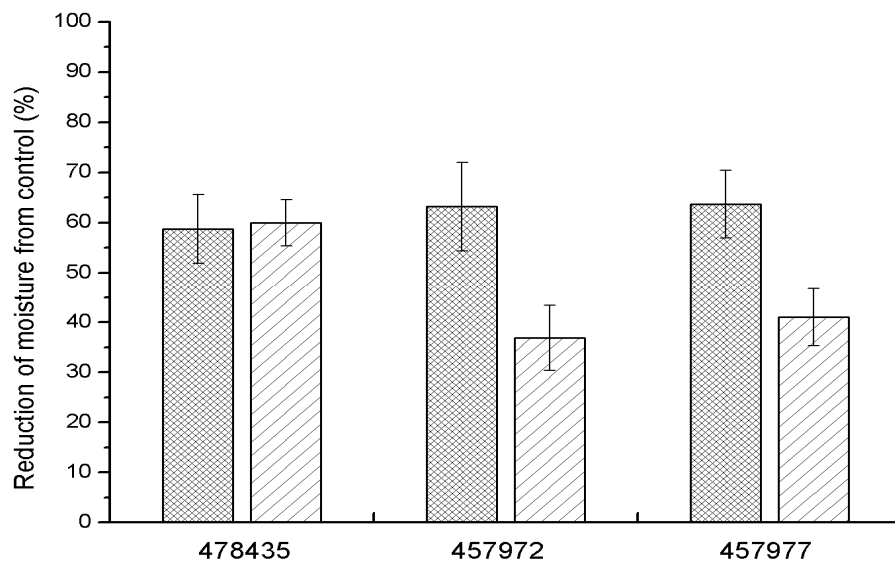


Figure 3.7. The effect of heat treatments on (A) flour moisture concentration, (B) moisture content per seed, and (C) the reduction of moisture content from control of *L. mutabilis* accessions 478435, 457972, 457977. Heat treatments are control (white), gradual (light shading), and sudden (dark shading). Error bars show ± 1 standard error.

Moisture content in flour was higher in heat stressed seeds than seeds from control ($P < 0.01$; Figure 3.7A). On the contrary, at per seed level, moisture content was much higher in the seeds of control than seeds of heat-stressed ($P < 0.01$; Figure 3.7B). Figure 3.7C indicated that moisture content of the seeds under sudden stress was equally reduced by about 60%; under gradual stress, both 457972 and 457977 had similar and lower value than 478435, but not significantly different.

Yield was shown to be positively correlated with seed weight ($r = 0.924$), oil mass in flour ($r = 0.908$) and per seed ($r = 0.958$), soluble sugar mass in flour ($r = 0.615$) and per seed ($r = 0.918$); and negatively correlated with moisture mass in flour ($r = -0.408$) and per seed ($r = 0.875$), ash mass in flour ($r = -0.866$) and per seed ($r = 0.608$). Although yield did not show a significant correlation with protein mass in flour ($r = -0.184$), at per seed level, the yield was strongly correlated with protein mass ($r = 0.909$).

On the per seed basis, all the components measured were reduced and highly correlated with yield

in all 3 accessions. Both sudden and gradual heat stresses reduced all the components in 3 accessions. Under sudden heat stress, all the component masses were reduced to an extent that was not significantly different between accessions. However, 457972 and 457977 responded differently to gradual heat stress than 478435. Protein mass, ash mass and seed weight were reduced significantly less in 457972 and 457977 than in 478435, and oil and soluble sugar mass in 457972 and 457977 were reduced less than in 478435, but not statistically significant.

Change of seed composition under heat stress resulted in the changes to flour composition. Both sudden and gradual stresses reduced seed weight, oil concentration and soluble sugar concentration in flour while ash and moisture concentration were increased. Gradual heat stress increased protein concentration in 3 accessions compared with sudden stress and control. Under sudden heat stress, flour compositions of the 3 accessions were not significantly different, except that 478435 had significantly higher ash concentration than 457972 and 457977 ($P < 0.05$). However, under gradual heat stress, 478435 had higher protein and ash concentration but lower soluble sugar concentration than 457972 and 457977 ($P < 0.05$).

4. Discussion

The results of this experiment have shown that both sudden and gradual stresses resulted in loss in yield and change in flour quality. In the control, the protein and oil concentration in the 3 accessions ranged respectively from 46% to 48%, and 10% to 13%. The protein data are within published values of 41% to 51% whereas the oil data are below the reported 14% to 24% (Jacobsen & Mujica, 2007), this could be attributed to different methods to extract oil or different environmental conditions. Ash concentration was 2.5% to 3%, soluble sugar 115 mg/g to 125 mg/g, and moisture 4% to 4.5%. The result of soluble sugar content in this experiment is much more than expected; it may be that other macro molecules were also included in the extracted solution. Accession 478435, with shorter vegetative phase duration, had higher protein and ash concentration but lower oil and soluble sugar concentration as well as lower yield compared with 457972 and 457977.

Sudden heat stress reduced seed mass at grain-filling stage more than gradual heat stress, as has been reported in cereals (Wardlaw, 1994) and oilseed rape (Aksouh et al., 2001), and the reduction of yield was due to the loss in every seed component. Under sudden heat stress (and under gradual stress), protein content per seed was reduced by 65% to 70% (40% to 55%), oil by around 85% (63% to 80%), ash by 35% to 50% (10% to 35%), and soluble sugar by 70% to 80% (55% to 80%). The difference of reduction in proportion resulted in quality change of flour, as the results showed that in heat-stressed flour, protein and ash were increased above control values. This is consistent with the results of studies focusing on effects of heat stress on yield and quality in cereals (Wrigley, 1994; Stone, 1995c; Stone, 1998; Spiertz et al., 2006), oil seed rape (Aksouh et al., 2001), and cotton (Hafeez ur Rahman Malik, S. A. & Saleem, 2004), and alkaloid concentration in different cultivars of *L. angustifolius* was also increased under high temperature (Jansen, 2009).

Wardlaw (1994) pointed out that the yield loss was partly attributed to shorter duration for grain-filling along with deactivation of enzymatic activity for photosynthesis and polymerization of macro molecules, such as starch and protein. Shorter duration for grain-filling could result from acceleration of senescence, as it was observed in this experiment that heat stress accelerated leaf fall and flower abortion. One explanation could be that heat stress can induce the production of ethylene (Taiz & Zeiger, 2006).

Seed weight was highly correlated with yield ($r=0.924$) and its stability under stress was associated with the reduction of protein, oil, soluble sugar mass similar to results from wheat

(Viswanathan & Khanna–Chopra 2001). This suggests that seed weight response can be regarded as a very important parameter when selecting the heat tolerant cultivars.

The breakdown of the equilibrium between respiration and photosynthesis rate also accounted for decrease of final yield and seed weight and size. Wardlaw (1994) proposed that photosynthesis and respiration are inhibited when the temperatures are above the temperature compensation point and CO₂ fixed by photosynthesis can not catch up with the carbon used as energy source for respiration. Finally, the kernel size and weight were reduced. Furthermore, the Respiration rate of rice under heat stress was shown to increase under high night-time temperature (Mohammed & Tarpley 2009). Therefore, it can be inferred that cultivars with longer vegetative phase duration are more tolerant to heat stress due to more CO₂ fixed.

Accession 478435, with its shorter vegetative phase duration, showed greater loss in every component than 457972 and 457977 under gradual heat stress. Sudden heat stress, however, failed to produce a comparable difference. Sudden stress in this experiment comprised a sharp rise from 22 °C to 38 °C within 15 minutes, then held for 4 hours, and hence may have severely damaged the synthetic processes as it was seen in this experiment that the protein content did not differ significantly between accessions. It may be that 38 °C, an arbitrarily–set temperature, was too high for this species, as different plants have different heat-stressing temperatures, for instance, sorghum is more heat-tolerant than many other cereals. Therefore, gradually rising temperature may better enable a plant to get acclimated to heat stress, and thus allows increased the resolution in screening heat-tolerant cultivars, similarly, Stone and Nicolas (1995) suggested that heat tolerance of different genotypes should be compared only in plants that are acclimated to the stress. Likewise, Aksouh et al. (2001) compared the effects of sudden and gradual heat stresses on the yield and quality of 3 canola cultivars with different vegetative phase duration, and found gradually–rising temperature showed higher resolution in screening tolerant genotypes. The design of increment of temperature should be similar to the local climate condition. Particularly, in cool-temperate areas, temperature is not likely to rise sharply within a short period; gradually rising temperature is more likely to simulate the ambient environment. In earlier studies, two different regimes were adopted to impose gradual treatment. In the first (Stone & Nicolas, 1995c; 1998), temperature was set to increase by a certain number of degrees per unit time and the results showed that gradually rising temperature could better enable plants to acclimate to heat stress and suggested that gradual treatment could be better in selecting heat tolerance. In the second (Aksouh et al., 2001), the gradual treatment was set to increase a number of degrees per day, and was

imposed for 4 hours in the middle of each day which is closer to real life compared with the first regime. Both showed better resolution in selecting heat tolerance.

It is interesting to note that the seed composition change of *L. mutabilis* under drought stress, is similar to the change in this experiment according to a study by Carvalho et al. (2005), where Drought stress was imposed on one cultivar of each *L. albus* and *L. mutabilis*. On the basis g/kg dry matter basis, crude protein, oil, crude fibre mass were reduced while the ash and total soluble carbohydrate mass were increased compared with well-watered plants. And a simultaneous study (Lizarazo, 2010) on screening drought-tolerant cultivars based on leaf temperature, stomata conductance, ion leakage measurement and water potential showed that 478435 was much more susceptible to drought stress than either 457972 or 457977. This supported the proposal by McDonald and Paulsen (1997) that selection for heat tolerance may improve drought tolerance since drought and heat stresses are interrelated with each other and often happen simultaneously in the field. Heat treatment can often be more precisely quantified and conducted more easily than drought treatment when growth chambers are available.

L. mutabilis could be a potential oilseed crop in northern part of Europe, where the climate is not hot, since its yield was shown to be very sensitive to hot climate and drought in southern and central Europe. It is necessary to screen accessions with tolerance to abiotic stresses. Short periods without rainfall or available irrigation system can expose the plants to drought and heat stress. Especially, like other lupin species, *L. mutabilis* has a potential role in phytoremediation in the contaminated soil where the irrigation system is not often available.

For prospective studies, investigation on the response of fatty acid and amino acid composition to heat stress is important to further elucidate the effects of heat stress on quality. It is important to improve the design or change the duration for imposing gradual heat treatment since gradual treatment showed better resolution in screening tolerant accessions. Due to the short growing season in areas of high latitudes, earliness is also an important factor. However, accession 478435 with the shortest vegetative phase was more susceptible to heat stress. Thus a wider range of genotypes should be investigated for different mechanisms for resisting stresses and better methodology for screening tolerant cultivars. Especially, it is important to seek heat- and drought-associated QTLs in order to adopt marker-assisted selection in screening and breeding programmes. In-depth investigation on the relationship between drought and heat stresses at phenotypic and gene-regulation level is important and necessary.

5. Conclusions

Heat stress is becoming more of a concern due to global warming and its relation with drought stress. It can reduce yield and affect crop quality significantly. Better understanding of the consequences of this trend will allow proper adaptation which will assist to reduce potential loss as in the future. Gradual heat treatment showed better resolution in screening heat tolerant accessions. In this experiment accessions with longer vegetative phase duration are more tolerant to heat stress. The tolerance, however, can not be attributed to longer vegetative growth alone; it is interesting to investigate other traits related to heat tolerance. Improving the design and adjustment of gradual treatment to local climate conditions could facilitate the domestication and reduce the potential loss of *L. mutabilis* in production.

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Appendix

Table 1. ANOVA table of the effects of heat stress on seed components on flour basis

Source	df	Total yield		Weight/50 seeds		Moisture in flour		Oil in flour		Protein in flour		Ash in flour		Soluble sugar in flour	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
Accession	2	57.75	P<0.01	0.37	NS	1.12	NS	12.84	P<0.05	14.60	NS	2.97	P<0.01	294.06	NS
Treatment	2	554.70	P<0.01	71.25	P<0.01	3.86	P<0.05	90.52	P<0.01	133.05	P<0.01	8.50	P<0.01	3177.52	P<0.01
accession*treatment	4	14.21	P<0.05	0.69	NS	0.89	NS	3.51	NS	11.74	NS	0.17	NS	987.58	NS
Error	18	4.49		0.49		0.59		1.71		9.57		0.19		379.56	

Table 2. ANOVA table of the effects of heat stress on seed components on per seed basis

Source	df	Oil per seed		Protein per seed		Soluble sugar per seed		Ash per seed		Moisture per seed		Weight per seed	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
accession	2	19.65	P<0.05	12.24	NS	8.88	NS	0.81	NS	0.423	NS	406.08	NS
treatment	2	584.39	P<0.01	6188.07	P<0.01	555.84	P<0.01	8.68	P<0.01	47.29	P<0.01	79171.12	P<0.01
accession * treatment	4	5.87	NS	63.91	NS	13.63	NS	0.54	NS	1.073	NS	770.51	NS
error	18	4.67		45.73		5.51		0.31		1.026		542.13	

Table 3. ANOVA table of seed composition reduction from control under gradual treatment

Source	df	Moisture reduction		Oil reduction		Protein reduction		Soluble sugar reduction		Ash reduction		Seed weight reduction	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
accession	2	457.80	NS	394.31	NS	216.57	P<0.01	596.36	NS	564.22	P<0.05	253.98	P<0.05
error	6	96.83		155.96		19.25		119.17		63.1		34.33	

Table 4. ANOVA table of seed composition reduction from control under sudden treatment

Source	df	Moisture reduction		Oil reduction		Protein reduction		Soluble sugar reduction		Ash reduction		Seed weight reduction	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
accession	2	22.5	NS	17.43	NS	56.69	NS	83.14	NS	373.82	NS	35.68	NS
error	6	170.19		28.02		82.7		102.9		341.9		75.37	

Table 5. ANOVA table of the effect of gradual heat stress on seed composition change on flour basis

Source	df	Moisture		Oil		Protein		Soluble sugar		Ash	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
accession	2	0.05	NS	15.26	NS	33.37	P<0.05	1724.84	P<0.05	1.70	P<0.05
error	6	0.44		3.82		4.84		278.87		0.17	

Table 6. ANOVA table of the effect of sudden heat stress on seed composition change on flour basis

Source	df	Moisture		Oil		Protein		Soluble sugar		Ash	
		MS	Sig	MS	Sig	MS	Sig	MS	Sig	MS	Sig
accession	2	1.58	NS	0.19	NS	1.43	NS	385.24	NS	1.053	P<0.05
error	6	0.78		0.72		19.21		759.19		0.191	